

Hepatoprotective Effects of Pomegranate against Methotrexate-Induced Acute Liver Injury: An Experimental Study

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Abstract

Background: Methotrexate (MTX) is a synthetic antimetabolite with a wide range of clinical applications, but its liver toxicity induced mainly through oxidative stress represents a primary concern on its clinical use. Pomegranate fruit contains many polyphenolic compounds that possess potent antioxidant effects and, therefore, have a possible hepatoprotective effect. **Objectives:** This study seeks to address the hepatoprotective effects of pomegranate against MTX-induced liver injury. **Materials and Methods:** Twenty-eight healthy female albino mice were grouped into four groups; control and MTX groups received oral 0.5 ml distilled water, while; the PG150 group received 150mg/kg oral pomegranate, and the PG300 group received 300mg/kg oral pomegranate. The oral course continues for 10 days, and on the last day, all groups were injected with 20 mg/kg MTX intraperitoneally, except the control group injected with normal saline. 48-hours later, samples were collected and prepared for biochemical and histopathological evaluation. **Results:** After biochemical analysis, MTX causes an elevation in serum Alanine aminotransferase, Lactate dehydrogenase, and liver tissue malondialdehyde, indicating hepatic injury, while pomegranate pre-treatment will hold down this elevation significantly and dose-dependently, causing amelioration of the toxic effect of MTX; the histopathological findings support this finding. Also, MTX causes consumption of liver tissue content of superoxide dismutase (SOD) and Glutathione (GSH), while pomegranate pre-treatment boosts the SOD and GSH hepatic tissue level. **Conclusion:** Pomegranate has a dose-dependent amelioration effect on the toxic effect of MTX on the liver.

Keywords: Methotrexate, pomegranate, hepatotoxicity, liver, oxidative stress

INTRODUCTION

Methotrexate (MTX) is a synthetic antimetabolite with many therapeutic indications, mainly anti-inflammatory and immunosuppressant at relatively low doses and antineoplastic at high doses.^[1,2] MTX competitively inhibits dihydrofolate reductase, so decreasing tetrahydrofolate level leads to blocks the synthesis of purines, pyrimidines, DNA, ribonucleic acid, thymidylate, and proteins.^[1-3]

It is well known that MTX is a hepatotoxic drug, and this leads to limitation of its clinical use; several mechanisms have been investigated for liver injury induced by MTX; the most accepted one is the loss of oxidative balance inside liver tissue and accumulation of reactive oxygen species (ROS), leading to oxidative stress-induced liver injury.^[4-7]

These ROS will cause lipid peroxidation reactions with intracellular and cell membrane lipids. ROS will also induce endoplasmic reticulum stress and more mitochondria dysfunction, and eventually, more ROS will be formed. MTX also decreases endogenous antioxidant activity by inhibiting the glucose-6-phosphate dehydrogenase enzyme, so it decreases nicotinamide adenine dinucleotide phosphate (NADPH) level, which is necessary for the formation of the reduced form of glutathione (GSH), which is responsible for scavenging ROS

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like hydrogen peroxide molecules.^[8-10] In addition, it is well known that ROS formation will induce autophagic cell death and apoptosis.^[11,12]

Pomegranate (*Punica granatum*, Punicaceae) is a fruit widely cultivated in different parts of the world; it was used as an anthelmintic and antidiarrheal agent until recently when the antioxidant activity of POM become more attractive. POM is rich in polyphenolic antioxidants, which include tannins, ellagic acid, gallic acid, and others.^[1,13-15]

Pomegranate and ellagic acid antioxidant properties have been proven on many occasions; this antioxidant effect is the primary mechanism for these agents to effectively ameliorate and prevent many diseases. Previous studies demonstrated the antioxidant properties of POM and EA and the effectiveness of these effects on the prevention of MTX-induced liver injury.^[1,16,17]

MATERIALS AND METHODS

Experimental design

Twenty-eight healthy female albino mice weighted $30 \text{ g} \pm 2$ were randomly collected after one-week adaptation period without any intervention; they are separated into four groups and placed into four sterilizer cages, each with seven mice and placed in a room of $21\text{--}25^\circ\text{C}$ temperature and 12-h light/dark cycles; also, a free excess to food and water was provided. The human care for animals was according to the guide and care of laboratory animals. These mice were separated into four groups [Figure 1] as follows:

1. Group 1: Control group ($n = 7$): Mice had received 0.5 ml of distilled water orally for ten consistent days, and on the 10th day, sodium chloride was injected intraperitoneally
2. Group 2: MTX group ($n = 7$): Mice had received 0.5 ml of distilled water orally for ten consistent days, and on the 10th day, MTX was injected intraperitoneally in a dose of 20 mg/kg
3. Group 3: Group PG150 ($n = 7$): Mice had received 150 mg/kg of pomegranate orally for ten consistent days,

and on the 10th day, MTX was injected intraperitoneally in a dose of 20 mg/kg

4. Group 4: Group PG300 ($n = 7$): Mice had received 300 mg/kg of pomegranate orally for ten consistent days, and on the 10th day, MTX was injected intraperitoneally in a dose of 20 mg/kg.

Rout and doses of the drugs were determined according to previous studies.^[18,19]

Serum sample preparation

At the 12 days, the mice were anesthetized using chloroform, and a 5 ml syringe was used to collect the blood from the heart; the collected blood was put in a gel and clot activator tube and left in the stand for 30 min, then centrifuged for 15 min at 5000 rpm at room temperature. The yielded isolated sample was stored in a freezer at -20°C to be used later.

Tissue sample preparation

After collecting the blood sample from the heart, the mice are sacrificed by cervical dislocation, and sharp scissors were used to isolate the liver. After this, the liver was weighed and divided into two pieces; one of them is 3-gram weighted piece prepared for homogenization by moving it to a Teflon homogenizer contain 1 ml of ice-cold (phosphate-buffered saline); after homogenization, homogenate will be centrifuged for 30 min at 3000 rpm, and the yielded supernatant isolated and stored at -20°C to be used later for biochemical analysis. At the same time, the remaining part of the liver was soaked in a 10% buffer formalin solution and stored at -20°C for later histopathological examination.

Measurement of liver function test

Serum levels of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured using an automatic analyzer (flexor-EL80, Vitalab, South Africa).

Measurement of liver tissue level of malondialdehyde (MDA), glutathione, and superoxide dismutase

After preparing the tissue homogenate, the double-sandwich enzyme-linked immunoassay (ELISA) technique is used

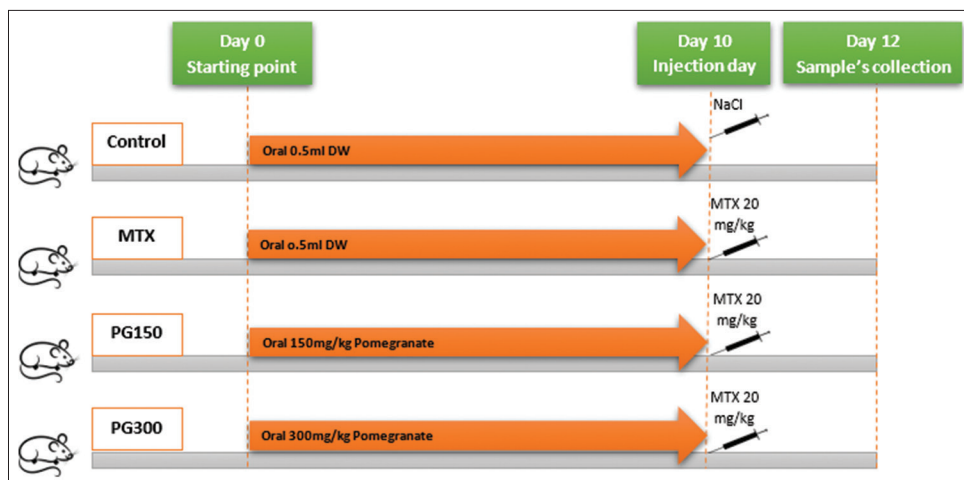


Figure 1: Scheme of the experimental design. Methotrexate, Methotrexate; NaCl: Sodium chloride, DW: Distilled water

to measure MDA, GSH, and SOD concentration in the supernatant for each marker from MyBioSource, USA, using an automated ELISA reader (ELISA-humareder®, Germany).

Histopathological analysis

Liver samples intended for histopathological studies were soaked in 10% buffer formalin, then the dehydration process was implied using graded (50%–100%) ethyl alcohol and embedded in paraffin wax. The liver tissue was then cut into small sections (4–5 micrometer thick), and these sections were stained with hematoxylin and eosin (H and E) stain for observation and photomicroscope assessment using a light microscope (M83MPTR-C140U; AmScope, California, USA). Histopathological assessment and scoring were done using the Ishak Modified HAI system, which depends on the extent of the histopathological changes,^[20,21] including degeneration of the hepatocyte and extent of the inflammatory reaction, and as follows:

1. Score 1, No abnormality
2. Score 2, Mild degenerative changes (10%)
3. Score 3, Moderate degenerative changes (25%)
4. Score 4, Severe degenerative changes (50%)
5. Score 5, Extensive and marked changes (>75%).

Statistical analysis

All statistical analyses were performed using SPSS (version 16) for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance with the *post hoc* test was used for data analysis. Expression of the data was as means and standard deviation values, and $P < 0.05$ was considered statistically significant.

RESULTS

Effect on oxidative stress biomarker (Glutathione, superoxide dismutase, and malondialdehyde) [Table 1]

Significant consumption of the antioxidant capacity represented by GSH and SOD was shown in the liver tissue after injection of MTX ($P < 0.001$) compared to the control group.

On the other hand, pretreatment with pomegranate causes significant elevation of tissue GSH level compared to the MTX group ($P < 0.001$) for both 150 and 300 mg/kg doses. Also, enhancement of tissue SOD level was observed ($P = 0.027$ and 0.717) in both groups PG150 and PG300, respectively [Table 1].

MDA as a by-product of oxidative stress was raised significantly ($P < 0.001$) after MTX injection in a dose of 20 mg/kg IP compared to the control group. Although, pomegranate pre-treatment significantly and dose-dependently held down the MDA level [Figure 2] with a P-value of ($P < 0.001$) for both groups.

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pomegranate pre-treatment significantly and dose-dependently held down the MDA level [Figure 2] with a P-value of ($P < 0.001$) for both groups.

Effect on the activity of serum marker enzymes (alanine aminotransferase and lactate dehydrogenase)

When MTX is injected into the MTX-only group, a significant elevation was observed in the serum ALT [Figure 3] and LDH level ($P < 0.001$) in respect to the control group [Table 2]. Pretreatment with pomegranate in both doses (150 and 300 mg/kg) will hold down this elevation significantly and dose-dependently when compared to the MTX group ($P < 0.001$ for both pomegranate groups).

Table 1: Effect of methotrexate and pomegranate on liver tissue level of oxidative stress biomarker (glutathione, superoxide dismutase, and malonaldehyde)

Parameter	Groups			
	Control	MTX	PG150	PG300
GSH (mcg/mL)	67.5±7.2	42.3±9.1*	67±5.4**	62.7±11.4**
SOD (U/mL)	467.9±36.5	64.3±36.6*	207.8±161.5**	88.4±23.8**
MDA (nmol/mL)	1.17±1.09	4.7±0.23*	1.37±0.51**	1.26±0.31**

* $P < 0.05$, significantly different from the control group, ** $P < 0.05$, significantly different from the MTX group, ^ $P \geq 0.05$, non-significant different with the MTX group, Data are presented as means±SD, $n=7$ for each treatment group. MTX: Methotrexate, PG: Pomegranate, GSH: Glutathione, SOD: Superoxide dismutase, MDA: Malonaldehyde, SD: Standard deviation

Table 2: Effect of methotrexate and pomegranate on serum level of enzyme biomarker (alanine aminotransferase and lactate dehydrogenase)

Parameter	Groups			
	Control	MTX	PG150	PG300
ALT (IU/L)	31.3±4.8	52.2±7.8*	35.29±7.6**	29.14±4.9**
LDH (ng/mL)	21.8±5.23	39.5±3.71*	17.13±7.4**	13.67±4.5**

* $P < 0.05$, significantly different from the control group, ** $P < 0.05$, significantly different from the MTX group, Data are presented as means±SD, $n=7$ for each treatment group. MTX: Methotrexate, PG: Pomegranate, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, SD: Standard deviation

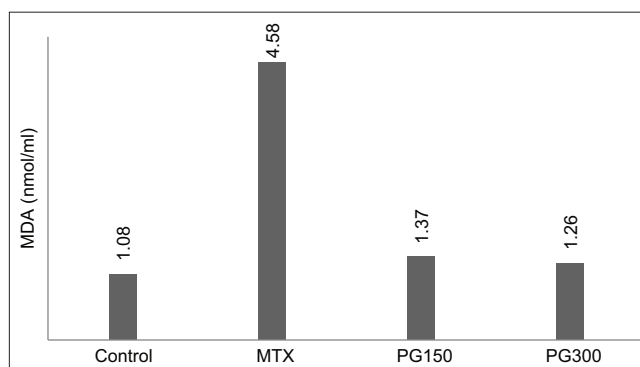


Figure 2: Malondialdehyde level comparison. Methotrexate, Methotrexate; PG: Pomegranate, MDA: Malonaldehyde

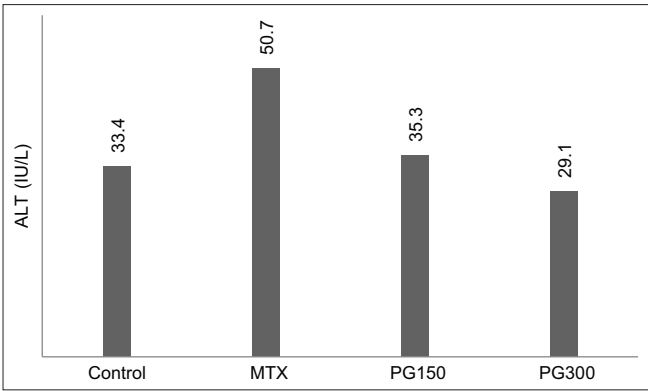


Figure 3: Aminotransferase level comparison. Methotrexate, Methotrexate; PG: Pomegranatem, ALT: Alanine aminotransferase

Table 3: Effect of methotrexate and pomegranate on liver tissue histopathological changes

Groups	Histopathological score	Degeneration changes
Control	1	No abnormality
MTX	5	Extensive and marked changes (>75%)
PG150	4	Severe degenerative changes (50%)
PG300	2	Mild degenerative changes (10%)

MTX: Methotrexate, PG: Pomegranate

Effect of methotrexate and different treatment regimens on liver tissue histopathological changes

As expected, the control group [Figure 4a] generally shows typical liver tissue architecture (Score 1) [Table 3]. On the other hand, the MTX group [Figure 4b] showed severe degeneration and inflammation of the liver tissue (Score 5).

Pretreatment with pomegranate has a positive effect on the degeneration of the hepatocyte, and this effect seems to be dose dependent. Group PG150 [Figure 4c] shows slight improvement on hepatocyte degeneration (Score 4), while more improvement was noticed (Score 2) in group PG300 [Figure 4d].

DISCUSSION

This study evaluates the hepatoprotective effect of pretreatment with different doses of pomegranate against MTX-induced liver injury. The result revealed that the potential of pomegranate to significantly and dose-dependently preserves the hepatic tissue from acute injury caused by MTX.

Injection of MTX in this study reduces both GSH level and SOD activity in the hepatic tissue. Direct inhibition of MTX on NADPH (NADPH is a cofactor for enzyme GSH reductase to make reduced GSH) together with overproduction of ROS, will cause a reduction in GSH cellular availability.^[22-25] Furthermore, the influence of MTX on Nrf2 expression might also reduce GSH tissue level.^[1]

SOD is an essential endogenous antioxidant enzyme, induces the conversion of mitochondrial generated ROS, mainly

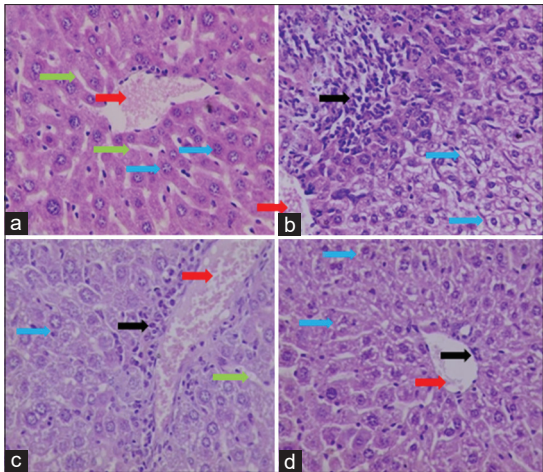


Figure 4: Photomicrograph of the liver mice histopathological section of the control group (a), Methotrexate group (b), group PG150 (c) and group PG300 (d). (a) the control group showed normal liver architecture with mild congested central vein (red arrow), normal hepatocyte (blue arrow), and normal sinusoids (green arrow). (b) Methotrexate group showed extensive inflammatory cells reaction (black arrow) with dilated and congested central vein (red arrow) and multifocal marked hepatocyte degenerative changes and cytoplasmic vacuolation (blue arrow). (c) Group PG150 showed dilated and congested central vein (red arrow) surrounded by moderate inflammatory cells reaction (black arrow) with dilated sinusoids (green arrow) and severe degenerative changes of the hepatocyte (blue arrow). (d) Group PG300 showed mild dilated and congested central vein (red arrow) with mild inflammatory reaction (black arrow) and mild hepatocyte degenerative changes (blue arrow). Magnification = ×40 and Haematoxylin-eosin staining of liver sections was used for all (a-d)

superoxide (O₂⁻), into less toxic hydrogen peroxide (H₂O₂) or molecular oxygen (O₂).^[26] On the other hand, MTX causes mitochondrial membrane damage, and more superoxide (O₂⁻) free radical production will lower SOD activity.^[20]

Overproduction of ROS and loss of endogenous antioxidants will shift the oxidative balance toward oxidative stress. In addition, ROS will cause mitochondrial dysfunction, and more ROS are produced, causing lipid peroxidation of the cell's lipid contents with MDA liberation.^[27] Therefore, in this study, MTX increases MDA hepatic tissue level more than four-fold compared to the control group.

In addition, both lipid peroxidation and mitochondrial dysfunction will eventually cause cell injury and damage to its membrane. This will make the cell lose its integrity, and the liver cell contents, significantly ALT and LDH, will be shifted outside the injured cell to the blood stream.^[28] Furthermore, neutrophil activation caused by ROS will cause augmentation of the cell injury.^[29]

Pomegranates contain many compounds (e.g., ellagic acid) with antioxidant and free radical scavengers' activity; this effect will counteract the excessive ROS released by MTX, preserving the level of both enzymatic and non-enzymatic antioxidants.^[20] Furthermore, pomegranate causes Nrf2 translocation from cytosol to the nucleus, which may cause

cytoprotective events and increase hepatic GSH and SOD levels as part of this event.^[1]

As a result of the positive pomegranate effect on GSH and SOD and enhancement of antioxidant capability in the liver tissue, pomegranate will ameliorate the oxidative stress caused by MTX and a reduction of ROS induce lipid peroxidation, which was reflected by the significantly lower level of MDA in the liver tissue compared to the control group.

Both lipid peroxidation and mitochondrial dysfunction caused by MTX will eventually led to cell injury and damage to its membrane; this will make the cell loses its integrity, and the liver cell contents, significantly ALT and LDH enzyme, will be shifted outside the injured cell to the blood stream.^[28]

Pomegranate preserves the oxidative balance inside the liver tissue, thus preventing hepatic injury, and this is manifested by the significant and dose-dependent reduction of ALT and LDH serum.

CONCLUSION

In this study, pomegranate significantly and dose-dependently ameliorates the hepatotoxic effect of MTX; this was evident by the significant reduction in ALT and LDH. Moreover, the histopathological finding supports the biochemical findings.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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